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Hetero-Nanoparticles by self-assembly of doxorubicin and cyclopamine conjugates

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Hetero-Nanoparticles by self-assembly of doxorubicin and cyclopamine conjugates

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ABSTRACT: The preparation of hetero-nanoparticles (NPs) with doxorubicin (DOXO) and cyclopamine (CYP) conjugates is presented. Biological evaluation on A431 cell lines confirms the maintenance of the activity of the parental drugs. The *in vivo* study shows that self-assembled NPs reduce tumour growth and toxicity of chemotherapy.

The use of self-assembled nanoparticles (NPs) is a challenging strategy that could result in a useful and smart approach to effectively treat cancer.¹ In particular, we have recently reported the preparation of a novel class of squalene conjugates with paclitaxel, podophyllotoxin, camptothecin and epothilone A.² The compounds obtained were characterized by a squalene tail that makes them able to self-assemble in water, and by a drug unit connected via a disulfide-containing linker to secure the release inside the cell. The need to trace the delivery of the nanoassemblies and to demonstrate the internalization of the drugs prompted us to prepare fluorescent hetero-nanoassemblies composed by a paclitaxel-squalene conjugate and fluorescein-squalene conjugate.³ Following the increasing interest of targeting cancer stem cells (CSCs)^{4,5} we moved forward and we demonstrated that cyclopamine – a hedgehog signaling pathway inhibitor – and paclitaxel conjugates self-assemble to generate hetero-NPs that show combined efficacy in the treatment of three different cancer cell lines. The use of ternary combination with the addition of a dye-squalene conjugate secured the obtainment of fluorescent NPs that allowed the cellular internalization by confocal microscopy and super resolution dSTORM.⁶ Besides the recent advances in cancer treatment, classical chemotherapy still exhibits two major pitfalls, high toxicity and limited efficacy possibly resulting in tumour resistance and subsequently cancer relapse.⁷ Pursuing our interest in self-assembled nanoparticles,⁸ we investigated

the combination of cyclopamine (CYP, Hedgehog pathway inhibitor⁴) and doxorubicin (DOXO) conjugates (Chart 1) with the aim to demonstrate the formation of hetero-NPs that could improve the performance given by the use of the simple parental drugs.

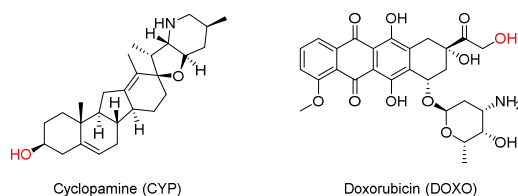


Chart 1. Structure of the used anticancer compounds. In red the anchor point for the functionalization.

Specifically, we decided to investigate the combination of cyclopamine-squalene (CYP-SQ) derivative **1**, a conjugate found to be active on different types of cancer cells,⁶ with doxorubicin-squalene (DOXO-SQ) **2**, described by Couvreur and some of us⁷ as able to improve the anticancer efficacy and the therapeutic index of DOXO (Chart 2).

NPs	Composition	Components molar ratio and concentration	Mean diameter (nm \pm S.D.)	Polydispersity index	Zeta potential (mV \pm S.D.)
NP0	CYP-SQ	10 μ M	127.8 \pm 0.723	0.106	+44.1 \pm 2.048
NP1	CYP-SQ/DOXO-SQ	1:1 (10 μ M, 10 μ M)	126.9 \pm 1.108	0.102	+61.6 \pm 4.18
NP2	CYP-SQ/DOXO-SQ	5:1 (10 μ M, 2 μ M)	129.4 \pm 1.589	0.109	+50.9 \pm 0.404
NP3	CYP-SQ/DOXO-SQ	10:1 (10 μ M, 1 μ M)	127.7 \pm 0.734	0.106	+56.8 \pm 2.66
NP4	DOXO-SQ	10 μ M	395.4 \pm 6.739	0.122	+58.1 \pm 0.643

Table 1. NPs composition and characterization

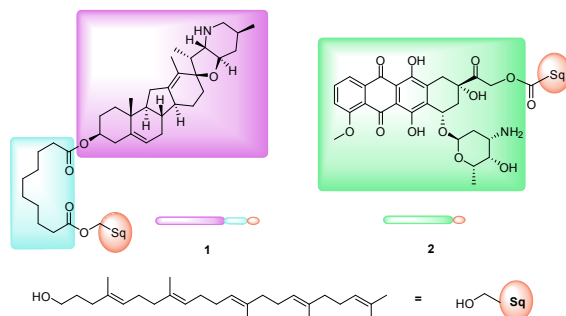


Chart 2. Structure of the drug-squalene conjugates CYP-SQ and DOXO-SQ.

Hetero-NPs were obtained by mixing two squalene conjugates, CYP-SQ (**1**) and DOXO-SQ (**2**) (Chart 2), prepared according to the previously described approaches.^{6,7} All NPs were prepared by solvent-displacement method,⁹ using THF as organic solvent and without adding any surfactant.

1 and **2** were co-nanoprecipitated at 1:1, 5:1, 10:1 molar ratios (Table 1, NP1, NP2 and NP3 respectively) with a constant concentration of **1**, giving a suspension of narrow monodispersed NPs. **1** and **2** were also separately nanoprecipitated to obtain NPs of CYP-SQ or DOXO-SQ alone (Table 1, NP0 and NP4). All the formulations were characterized in terms of mean diameter and zeta potential of the NPs. As showed in Table 1, CYP-SQ and DOXO-SQ alone spontaneously self-assembled in water forming NPs; however, DOXO-SQ NPs displayed a larger mean diameter of about 400 nm. When DOXO-SQ was formulated with an equimolar amount of CYP-SQ (NP1), the mean size drastically reduced from 400 to about 130 nm; in these conditions DOXO-SQ probably took a different supramolecular organization and mutual interactions between the two squalene conjugates caused the formation of smaller nanoparticles. The addition of DOXO-SQ in various molar ratios did not influence the mean size of CYP-SQ NPs. The zeta potential of all NPs is highly positive; for NPs containing DOXO-SQ it is higher than the one of CYP-SQ-containing NPs. As reported in the Supporting Information, after 4-weeks storage at 4°C in water, no appreciable NPs size and/or zeta potential changes were detected by Dynamic Light Scattering (DLS) for all formulations (except for DOXO-SQ NP mean diameter) and no NPs aggregation was observed. After dilution with fetal bovine serum, NP1 did not show aggregation, even after 24 h at 4°C. On the contrary, the incubation in PBS 10 mM resulted in an increase of the NP1 mean diameter up to about 400 nm (700 nm after 24 h).

We next investigated in details the effect of the NPs in a cellular system¹⁰ with an activated Hedgehog pathway. Specifically, we used the human epidermoid carcinoma cell line A431,

which carries a single point mutation in exon 23 of both alleles of *Patched1* gene, resulting in a proline to leucine substitution and subsequent constitutive activation of the Hedgehog pathway.¹¹ In a similar experimental setting, we studied the inhibition of proliferation and the apoptosis induction upon addition of CYP, DOXO and distinct ratios of CYP:DOXO, conjugate **1**, conjugate **2** and distinct ratios of **1** and **2**, free or assembled in NPs.

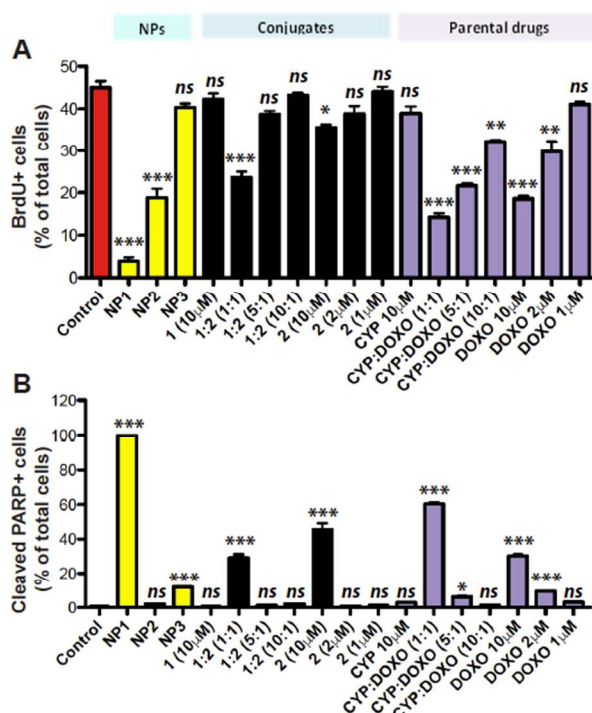


Figure 1. Effect on proliferation (A) and viability (B) in the A431 cancer cell line of cyclophosphamide, doxorubicin (reported as Parental drugs), compounds **1** and **2** not nanoprecipitated (reported as Conjugates) and nanoprecipitated compounds **1** and **2** (reported as NP). Concentration of the samples: NP1 (nanoprecipitated CYP-SQ = DOXO-SQ = 10 μ M); NP2 (nanoprecipitated CYP-SQ = 10 μ M, DOXO-SQ = 2 μ M); NP3 (nanoprecipitated CYP-SQ = 10 μ M, DOXO-SQ = 1 μ M); **1:2** (1:1): CYP-SQ = DOXO-SQ = 10 μ M; **1:2** (5:1): CYP-SQ = 10 μ M, DOXO-SQ = 2 μ M; **1:2** (10:1): CYP-SQ = 10 μ M, DOXO-SQ = 1 μ M; CYP:DOXO (1:1): CYP = DOXO = 10 μ M, CYP:DOXO (5:1): CYP = 10 μ M, DOXO = 2 μ M, CYP:DOXO (10:1): CYP = 10 μ M, DOXO = 1 μ M. Statistical significance (Student's t test) compared to Control: ns, $p > 0.01$; *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

DOXO alone at 10 μ M (Figure 1A) was able to inhibit proliferation of more than 50%, and the addition of CYP further enhanced the cytostatic effect. The combination of **1** and **2** in 1:1 molar ratio also resulted in inhibition of proliferation, although to a lesser extent. Interestingly, when NP1 was used, cell proliferation is essentially entirely blocked. The same trend ap-

pears when investigating cell death, with CYP and derivative 1 not causing significant apoptosis, DOXO and derivative 2, alone or in combination with CYP or derivative 1 inducing apoptosis in 30-60% of the cells, while when assembled in NP1, practically all cells are dead (Figure 1B). Notably, while apoptosis is only observed at the 1:1 ratio of CYP:DOXO or of derivatives 1 and 2, inhibition of proliferation could be observed also at the ratio 5:1, potentially indicating that either lower concentration of DOXO or derivative 2 is necessary for the cytostatic function, or that apoptosis is a later stage event in this system. A time course analysis of proliferation and apoptosis would discriminate between the two options, but this is out of the scope of this work.

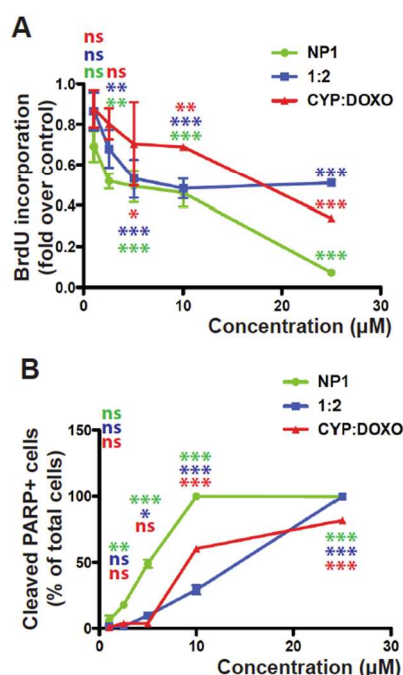


Figure 2. Dose response of equimolar concentrations of CYP and DOXO as parental drugs (CYP:DOXO), conjugates (1:2) or assembled in nanoparticles (NP1). The effect on proliferation (A) and their cytotoxic potential (B) is estimated by FACS analysis of BrdU incorporation and cleaved PARP immunostaining respectively. Concentration of the samples: NP1 (nanoprecipitated CYP-SQ = DOXO-SQ = 10 μM); 1:2 (1:1): CYP-SQ = DOXO-SQ = 10 μM; CYP:DOXO (1:1): CYP = DOXO = 10 μM. Statistical significance (Student's t test) compared to the control: ns, $p > 0.01$; *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

Given the strong cytostatic and cytotoxic effect of the 10 μM equimolar concentration of CYP:DOXO and 1 and 2, free or assembled in NP1, we sought to investigate the dose response of A431 cells to these formulations, so as to define the lowest concentration required for a toxic effect. As shown in Figure 2, NP1 has a significant cytotoxic and cytostatic effect already at 2.5 μM, while the free drugs require at least 3-fold higher concentration to induce apoptosis. Small differences in the percentage of proliferating cells treated with 10 μM of compounds in the sets of experiments presented in the figures 1A and 2A are a result of difference in confluence, since the first set of experiments was performed in 6-well plates and the latter in 24-well plates. To conclude, our results show that the combination of the compounds 1:2 assembled in NP1 exhibit strong cytotoxic and cytostatic effect on cells requiring Hedgehog signaling even at low concentrations.

Given the very promising results obtained by the *in vitro* experiments using cell lines, we sought to investigate whether these results could also be observed *in vivo*, now investigating both the antitumoral activity and the toxicity of the formulation. We used a xenograft model, injecting A431 cells into the flank of immunosuppressed mice, as detailed in Supporting Information. When tumours reached 3 mm in diameter, mice were administered intraperitoneally twice weekly equimolar concentrations (4 mg/kg of derivative 1 and 5mg/kg of derivative 2) of 1, 2, and 1:2 free or assembled in NP1, or solely the vehicle. Strikingly, when the derivative 2 is injected as a free drug, alone or together with 1, it induces very high toxicity and the mice die or have to be sacrificed due to weight loss and general health deterioration less than a week after treatment initiation (Figure 3A). The derivative 1 alone is not toxic, but does not have an effect on tumour growth. Although, when the same concentration of 1:2 is administered as self-assembled NPs, there is no toxicity and there is a significant delay in tumour growth compared to the control mice (Figure 3B).

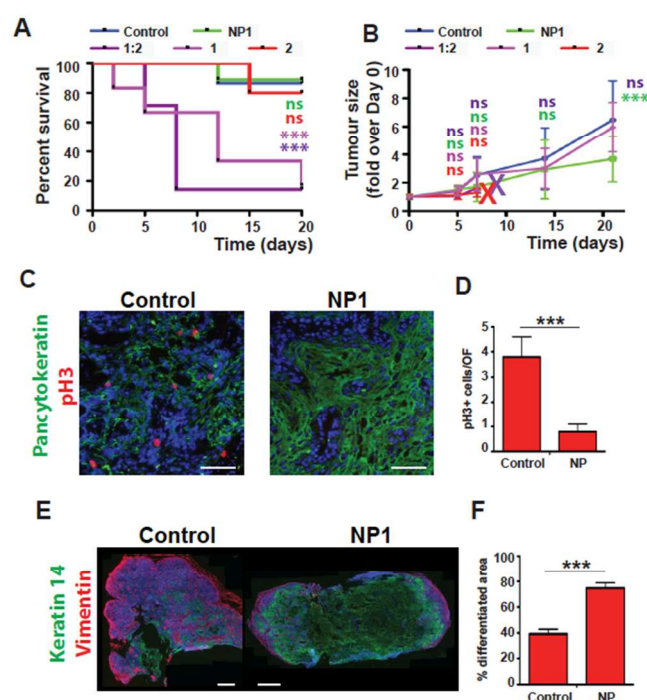


Figure 3. In vivo effect of equimolar concentration of CYP and DOXO conjugates, alone or in combination, or assembled in nanoparticles (NP1). (A) Kaplan Meier survival plot of mice upon treatment-administration, as indicated, twice weekly. Note that conjugate 2 alone or in combination with conjugate 1 exhibits high toxicity. (B) Follow up of tumour size over time, upon treatment as described in (A). 20 random images per tumour form 3 separate tumours were counted. (C) Representative immunofluorescence images of tumours from mice treated with NP1 or vehicle, stained for p33 labeling cells undergoing mitosis. Note the inhibition of proliferation upon treatment with NP1. The antibodies are colour-coded. (D) Histogram illustrating the quantification of proliferating cells in mice treated with NP1 or vehicle, as illustrated in (C). Results are expressed as p33+ cells per optical field. (E) Representative mosaic immunofluorescent images of tumours from mice treated with NP1 or vehicle, stained with Keratin 14 indicating differentiated cancer cells and vimentin labeling tumour-associated fibroblasts and cells undergone epithelial-mesenchymal transition. Note the striking difference in the differentiation of the tumours. The antibodies are colour-coded. (F) Histogram illustrating the quantification of differentiated areas in tumours from mice treated with NP1 or vehicle, based on the expression of Keratin 14. 20 random images per tumour form 3 separate tumours were counted. Concentration

of the samples: NP1: CYP-SQ = DOXO-SQ = 10 μ M; 1:2(1:1): CYP-SQ = DOXO-SQ = 10 μ M; 1 = 10 μ M; 2 = 10 μ M. Statistical significance (Student's t test): ns, $p > 0.01$; ***, $p < 0.0001$. Scale bars: Panel B, 100 μ m; Panel E, 1000 μ m.

Microscopic analysis of tumours from control mice and mice treated with NP1 3 weeks after treatment showed that the latter exhibit very strong inhibition of proliferation (Figure 3C, D), and a more differentiated phenotype (Figure 3E, F). The latter could have important implications in moderating the metastatic potential, although longer experiments should be performed to investigate this possibility. Therefore, our *in vivo* experiments unambiguously show that self-assembled NPs reduce tumour growth and toxicity of chemotherapy; this could enable the administration of higher doses, thus more effective cancer treatment.

In this paper we reported the formation of hetero-NPs combining cyclopamine- and doxorubicin-squalene derivatives. DLS characterization demonstrated that NPs are narrow monodisperse and stable for 4 weeks. *In vitro* biological evaluation on confirmed that the cytotoxic activity of the parental drugs is maintained. The efficacy of the nanoformulated drugs is higher than the single combination of the conjugates. Importantly, the *in vivo* experiments showed that cyclopamine-doxorubicin NPs not only reduce tumour growth, but also significantly decrease the toxicity of chemotherapy in mice. This will allow the administration of higher doses of chemotherapy, potentially eliminating the more resistant tumour cells and therefore leading to more effective treatment.

The results obtained demonstrated that the approach of the self-assembled hetero NPs is a promising way to treat different types of cancer cells and it makes possible to modulate the ratio of the combined drugs. Moreover, these results point out the possible application of this approach in the personalized therapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. *These authors contributed equally.

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